Original Article

Reactive oxygen species (ROS) mediates non-freezing cold injury of rat sciatic nerve

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Abstract: Non-freezing cold injury is an injury characterized by neuropathy, developing when patients expose to cold environments. Reactive oxygen species (ROS) has been shown as a contributing factor for the non-freezing cold nerve injury. However, the detailed connections between non-freezing cold nerve injury and ROS have not been described. In order to investigate the relationship between non-freezing cold nerve injury and reactive oxygen species, we study the effects of two cooling methods—the continuous cooling and the intermittent cooling with warming intervals—on rat sciatic nerves. Specifically, we assess the morphological changes and ROS production of the sciatic nerves underwent different cooling treatments. Our data shows both types of cooling methods cause nerve injury and ROS production. However, despite of identical cooling degree and duration, the sciatic nerves processed by intermittent cooling with warming intervals present more ROS production, severer reperfusion injury and pathological destructions than the sciatic nerves processed by continuous cooling. This result indicates reactive oxygen species, as a product of reperfusion, facilitates non-freezing cold nerve injury.

Keywords: Reactive oxygen species, sciatic nerve, cold injury, intermittent cooling, continuous cooling

Introduction

As a common result of exposure to cold environments, cold-induced nerve injury could happen in patients undergoing cryotherapy and individuals engaged in sport-related or work-related activities in cold, wet, or windy conditions. Patients could suffer devastating nerve damage after using a cold therapy machine or engaging themselves in physical activities in cold environments for too long [1].

The effects of cold ambient temperature on peripheral nerves are complicated and have not been fully studied. Previous studies point out the effects of coldness on the nerve fibers are selective and region restricted. One theory to explain this selectiveness was proposed by Denny-Brown and his colleagues who suggest that cold-induced nerve lesions might be primarily ischemic in origin [2]. Similarly, two hind limb cold-immersion studies, fulfilled by Kennett, Gilliatt [3] and Irwin [4], describe the site of severe injury is largely confined to a restricted region of the upper tibial nerve just below the water line identified as “warm-cold interface”. Additionally, a later study performed by Jia and Pollock shows non-freezing cold nerve injury is enhanced by intermittent cooling, implying the local ischemia could aggravate nerve injury [5]. These findings indicate that the cycles of reperfusion occurred at the “warm-cold interface” interface could be causal for cold nerve injury. However, the molecular mechanisms of how reperfusion intensifies cold injury have not been concluded [5, 6]. Jia and his colleagues proposed that the pathological basis of non-freezing cold nerve injury is multifactorial which includes intravascular aggregation, disorder in blood flow, endothelial damage and thrombotic occlusion. Given reactive oxygen species (ROS) has been proved to be associated with myocardial ischemia and other reperfusion injury, it is reasonable to question whether ROS is one of the factors associated with cold nerve injury [5]. In this paper, we reasoned whether ROS contributes to the pathogenesis of non-freezing cold nerve injury. Specifically, we evaluated the morphological changes of cold-injured sciatic nerves and mea-
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...sured their total ROS production as well as lipid peroxidation levels. We also compared the damage severity of the injured sciatic nerves exposed to either continuous cooling or intermittent cooling methods.

Materials and methods

Animals

All animals used in this project were provided by Capital Medical University. Rats were kept on a light-dark cycle (12 h/12 h) with lights on at 6:00 am and were given unlimited access to food and water. All experiments were conducted according to the Institutional Animal Care and Use Committee of Laboratory Animal Science, Capital Medical University. Total of 64 male Wistar rats, weighing between 300 to 350 grams, were randomly divided into the continuous cooling group and the intermittent cooling group. One side of the rat sciatic nerves was cooled according to experimental exposure, the other side received sham operation. The cooling process has been previously described [4]. The sciatic nerves of rats assigned to the continuous cooling group were immersed in 4°C for 2 hours while the sciatic nerves of rats assigned to the intermittent cooling group were exposed to 4°C for 1 hour followed by a rewarming process at 37°C for 1 hour before they were immersed in 4°C for another 1 hour. The sciatic nerves from both groups were sampled and analyzed at same time points after the surgeries were done. Time points we sampled at included “immediate after cooling”, “4 hours after cooling”, “1 day after the surgery” and “3 days after the surgery”. Anesthesia was achieved by an intraperitoneal injection of 20% pentobarbital sodium (0.25 ml/100 mg) repeated at half of this dosage every 20-30 min to maintain adequate anesthesia. Once a rat was fully anesthetized, its right sciatic nerve was exposed by a lateral incision around the mid-thigh region. In order to put the cooling device beneath the sciatic nerve, approximately 20 mm of the sciatic nerve was mobilized proximally to the branching site of posterior tibial and common peroneal. Care was taken to avoid injuring other tissues, such as vessels and muscles. The ipsilateral tibial nerve was then exposed at the ankle level for recording purposes.

Apparatus

In order to achieve local cooling of the sciatic nerves, we used a small copper cuff, shaped it into a hollow semicircle and coated it with epoxy resin [7]. Water inlet and outlet tubes were separated at a distance of 10 mm. The nerve temperature was monitored by a thermistor attached to base of the cuff. A second thermistor was placed gently on the surface of the sciatic nerve but the opposite side of the cuff. Once the cuff was correctly positioned to the origin of the inferior gluteal nerve, experimenters started to monitor the temperature readings and assured the difference between the two thermistors did not exceed 0.5°C. Paraffin (liquid paraffin BP) was applied to the nerve in the end of the experiment by infiltrating it into the area between the cuff and underlying muscle. The local cooling was achieved by a water/ethylene glycol mixture.

Measurement of reactive oxygen species production

1.5 centimeters of a sciatic nerve were sampled immediately after cooling process ended, 4 hours after cooling ended, 1 day or 3 days after cooling ended. After sampled, the sciatic nerves were immediately immersed in cold saline solution and wiped with filter papers on an ice-cold plate. Electron spin resonance (ESR) technique was applied to examine the samples. Particularly, ESP-300 spectrometer (Bruker, Germany), with the parameters of 10 mw power, 100 kH modulation, 1 mT amplitude and 335 mT central magnetic field was used to analyze the samples placed in a quartz tube at room temperature. Each test was repeated four times.

Malondialdehyde (MDA) analysis

The collected sciatic nerve tissue samples were rinsed with cold saline solution, blotted and preserved at -196°C before the MDA assay was performed. Before the test started, tissue was then homogenized in 0.1 mol/L phosphate-buffer (pH 7.4) at 4°C using homogenizer (ULIRA-TURRAXT18, VWR, USA). The homogenates were then centrifuged at 4000 rpm for 10 min. The supernatants were collected and used for malondialdehyde (MDA) analysis. MDA
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Electron microscopy

The sciatic nerve tissues were fixed by being immersed in 2.5% glutaraldehyde solution at 4°C for 2 hours. All samples were further fixed with OsO₄, dehydrated in alcohol gradients and embedded in Epon 812 reagents. Semi-thin sections (1 µm) were stained with toluidine blue and observed by light microscopy. The ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate. All sections were examined and photographed by an EM 208 s EX transmission electron microscope (Philip, Germany).

Statistical analysis

All results are expressed as mean ± standard deviation. Data were analyzed using the unpaired two-tailed student’s t test. P values of P < 0.05 were considered significant. Statistical analysis software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for all analysis.

Results

Morphological changes of cool-injured sciatic nerves

To examine and verify non-freezing cool injury was successfully induced in our rat model, we performed electronic microscopy in all sciatic nerve samples. These nerve samples were collected at different time points after the sciatic nerves were exposed either to continuous cooling or intermittent cooling. The morphology of myelinated fibers confirmed all the sciatic nerves exposed to treatments were injured but the level of injury differs. The myelinated fibers of sciatic nerves, sampled immediately after continuous cooling treatment ended, were relatively well preserved with occasional occurrence of swollen axons (Figure 1E). However, the sciatic nerves exposed to intermittent cool-
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![Graph A](image1.png)

**A. ROS Production in Continuous Cooling Group**

ROS production in the continuous cooling group sampled four hours after cooling treatment ended, showed swollen and loosed myeline sheathes as well as partial degradation of the microfilaments and microtubules while the nerves in the continuous cooling group at same time point only exhibited moderate myeline sheath degeneration (Figure 1B, 1F).

![Graph B](image2.png)

**B. ROS Production in Intermittent Cooling Group**

Sampled one day after the cooling treatment ended, the sciatic nerves in both continuous and intermittent cooling group showed degeneration of globular structures. But the nerves in intermittent cooling group also presented blurred myeline sheath layers and partial mitochondrion cavitation (Figure 1C, 1G).

Moreover, 3 days after cooling treatment ended, the sciatic nerves in the intermittent cooling group showed advanced axon demolitions and emptiness while the sciatic nerves in the continuous cooling group only displayed partial axon demolitions (Figure 1D, 1H). Taken together, both continuous and intermittent cooling caused damage in the sciatic nerves. Nonetheless, severer nerve impairments were observed in the sciatic nerves in intermittent cooling group.

Intermittent cooled sciatic nerves produce more reactive oxygen species

![Graph C](image3.png)

**C. ROS Production**

Based on the observation of mitochondrion destruction in the previous experiments, we further designed experiments to seek mitochondria-related explanations for the nerve

**Figure 2.** ROS production of sciatic nerves exposed cooling treatment. A. The ROS production of the sciatic nerves in continuous cooling group **P < 0.01 (n=6).** B. The ROS production of the sciatic nerves in intermittent cooling group **P < 0.01, *P < 0.05 (n=6).** C. Comparison of the ROS production between two cooling groups *P < 0.05 (n=6).**
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degeneration happened in cold injury. In order to test the hypothesis that rewarming reperfusion-originated reactive oxygen species (ROS) deteriorates cold injury, we compared the ROS production in rat sciatic nerves exposed to either the continuous cooling or intermittent cooling conditions. This comparison further allowed us to reason whether the reperfusion occurred during cooling processes would worsen cold injury. The sciatic nerves in both groups received the same amount of cooling time, except for the one hour additional 37°C rewarming process for sciatic nerves in the intermittent cooling group. We measured and compared the ROS production within each group at different time points. We also compared the ROS production between two groups at same time points. Immediately after cooling treatment ends, the ROS production of sciatic nerves in continuous cooling group did not reach statistically significant level compared with sham control, considering as a “no-reflow” phenotype described by Jia and his colleagues [5]. However, the induction of the ROS level right after continuous cooling ended supported the idea that reperfusion did happen in continuous cooling condition at a minor level. Overall in continuous cooling group, the reactive oxygen species production gradually increased after cooling ended, peaked at 1 day after the continuous cooling treatment ended and then went downward in 3 days after the treatment ended (Figure 2A). This post-injury ROS production could be explained by the rewarming and reperfusion processes after the cold injury upon the sciatic nerves. Similar pattern of ROS production was observed in the intermittent cooling group. The experi-

**Figure 3.** MDA production of the sciatic nerves exposed cooling treatment. A. The MDA level of the sciatic nerves in continuous cooling group \( **p < 0.01 \) (n=6). B. The MDA level of the sciatic nerves in intermittent cooling group \( **p < 0.01, *p < 0.05 \) (n=6). C. Comparison of the MDA level between two cooling groups (n=6).
mental sciatic nerves in the intermittent cooling group produced more ROS than the controls and, likewise, the peak of ROS production was observed 1 day after the cooling treatment ended (Figure 2B). Notably, under the intermittent cooling condition, the ROS production of the sciatic nerves right after coldness exposure was significantly higher than the control’s, indicating reperfusion happened during the warming hour contributed to the ROS production and possibly reinforced damage on nerves. The sciatic nerves exposed to coldness in the intermittent cooling group produced more ROS than the ones in the continuous cooling group at all sampled time points, indicating intermittent cooling condition indeed caused severer damage in the sciatic nerves (Figure 2C). Taken together, we have provided evidence to support the idea that although reactive oxygen species produces at a basal level under continuous cooling condition, the warming process on the nerves could intensify ROS production and further damages the nerves.

**Malonic Dialdehyde (MDA) elevated after reperfusion injury**

Malonic Dialdehyde (MDA) is produced as a product of lipid peroxidation and a metabolic product of arachidonic acid whose level is correlated with ischemia [9, 10]; MDA level has been used as an indicator for the ROS induced lipid peroxidation and tissue damage. High MDA level indicates severe tissue injuries including cold injury [11, 12]. In order to determine the existence of reperfusion caused nerve injury, we performed MDA assay on the sampled sciatic nerve tissues. MDA level of the sciatic nerves exposed to either continuous or intermittent cooling treatment maintains at a stable level when the sciatic nerves were undergoing cooling process. The production of MDA happened after rewarming in both groups (Figure 3A, 3B). Specifically, in the continuous cooling group, the MDA level of the sciatic nerves maintained high starting from 1 day after cooling treatment ended. The MDA level of the sciatic nerves in intermittent cooling group first peaked at 1 day after cooling treatment ended but decreased at 3 days after cooling treatment ended. The MDA level of the sciatic nerves was not statistically significantly different between the continuous cooling group and the intermittent cooling group in all the time points after rewarming (P > 0.05) (Figure 3C). However, this result supports the idea that non-freezing cold injury on sciatic nerves is ischemic origin. The cold injury could worsen if the rewarming from surrounding environment causes reperfusion upon sciatic nerves. Also, since the produced MDA during cooling treatment did not differ between two groups, the intensified damage observed in the sciatic nerves of intermittent cooling group was not likely to be a result of the ROS related MDA production, indicating the involvement of other ROS related mechanisms.

**Discussion**

Multiple investigations have confirmed that, initial tissue damage could be reversible in non-freezing cold injury, but rewarmin process upon tissues results in irreversible damage to tissues. Jia and his colleagues [5, 6] suggest that although the sciatic nerves in both continuous cooling and intermittent cooling conditions are exposed to coldness at the same intensity and duration, nerve damage is much more severe when the nerves are cooled intermittently rather than continuously. This difference in pathological feature suggests that the nerves cooled intermittently are potentially exposed to reperfusion cold injury and their severe tissue damage could be contributed by the reactive oxidative species produced during the reperfusion process. These reactive oxygen species actively interacts with proteins, lipids, carbohydrates and nucleic acids, causes peroxidation of the double bonds between carbon atoms in the lipids, cross-links sulfhydryl radicals of amino acids and eventually damages membranes, fragmentize peptides and disorganize DNAs [13]. Nagamatsu [14] demonstrates that an increase in the ROS generation in nerve ischemia is accompanied with the breakdown of blood-nerve barrier, endoneurial edema, increases in hydrogen peroxide and ischemic fiber degenerations [15].

In this paper, we report elevated cold injury in sciatic nerves treated by intermittent cooling condition compared with continuous cooling condition in terms of morphological changes and ROS production. Similar with previous studies, we observe slightly elevated reactive oxygen species production immediately after cooling treatment ended in both cooling models, supporting the argument that a small scale of reperfusion happens in the injured site during
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coldness. Based on our morphological observations, we also reconfirm the observation that intermittent cooling treatment causes more disastrous injury to the nerves than continuous cooling treatment. We further prove the deteriorated injury of the sciatic nerves exposed to intermittent cooling condition is due to the reperfusion mediated ROS production during the rewarming process. The cooling process generates hypoxia condition and as the injury gets recovered, reperfusion brings oxygen back to the injury sites where oxidation and anti-oxidation system occurred unbalanced, allowing the generation and migration of reactive oxygen species across the endothelium of the vasa nervorum which further damage the adjacent nerve fibers [16].

Based on the fact that we detected decreasing reactive oxygen species level 3 days after cooling treatment ended, deeper investigations can be focused on degradation of reactive oxygen species after reperfusion and whether the ROS produced in cold injury is correlated with the recovery of nerve cold injury. Also in this paper, we propose and demonstrate MDA production could be one of the explanations for nerve damage caused by reactive oxygen species. However, the insignificant changes of MDA level between the two cooling models indicates other potential ROS related mechanisms may involve in the pathogenesis of cold nerve injury.

In summary, the method of repetitively cooling on nerves accelerated ROS production because of ischemia-reperfusion process happened during the cooling treatment. Detailed mechanistic studies about how ROS involves in the formation of the non-freezing cold injury could shed lights on the development of therapies to treat cold nerve injury.

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Disclosure of conflict of interest

None.

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